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Botrychium ascendens W. H. Wagner (Ophioglossaceae) in Newfoundland and Notes on its Origin

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Abstract.—*Botrychium ascendens* is reported from Fogo Island in Newfoundland as an addition to the flora of the province. Fogo Island plants are identical to plants in western North America, including those from the type locality, in comparisons of leaf morphology, spore size, and allozyme expression. Comparisons are made with related and confusing taxa, *B. campestre*, *B. crenulatum*, *B. lineare*, and the American genotype of *B. lunaria*. Newfoundland plants display a high level of fixed heterozygosity and large spore size, indicating they are allotetraploid, and supporting suggestions that *B. ascendens* is derived from ancient hybridization between *B. crenulatum* and the *B. lineare/campestre* complex. The current distribution of *Botrychium ascendens* and its putative parents suggest it probably originated in western North America and migrated across northern Canada to Newfoundland.

Key Words.—*Botrychium ascendens*, leaf morphology, allozymes, taxonomy

Canada's Maritime Provinces of Nova Scotia, New Brunswick, Newfoundland and Québec are reported to harbor 14 species of *Botrychium* (Cody and Britton, 1989; Wagner and Wagner, 1993; Kartesz, 1999), not including *Botrychium ascendens* W. H. Wagner, a species commonly found throughout western North America, east to central Alberta, with an outlier collection from northern Ontario (Wagner and Wagner, 1993).

Wagner and Wagner (1990) tentatively reported a 1985 Britton and Anderson collection from Fogo Island in Newfoundland as *Botrychium campestre* W.H. Wagner & Farrar. The Wagners were uncertain because the pressed material was scanty. They wished to see a larger collection to study the morphological variation in the population. A traditional problem in the elucidation of species in *Botrychium* subgenus *Botrychium* is that much herbarium material consists of only one to a few plants, these often folded or shriveled, and thus difficult to classify. The most useful samples have leaves pressed with all pinnae flat and clearly visible, and have a minimum of 10–20 plants showing the variability within the population. (Underground parts are not diagnostic in moonwort species identification. Carefully harvesting the above-ground leaf by cutting at ground level allows the below-ground bud to produce new leaves in subsequent years.) Limited samples fail to show typical population variation, and if the individual specimens are small, they may resemble juvenile or small plants of other taxa. Diagnostic features observable in the field, such as stature, color, fleshiness and luster, are rarely noted by collectors on museum labels.

To overcome these difficulties, we visited the Fogo Island population to secure an adequate sample.

Wagner and Wagner (1986) reported a chromosome number of n = 90 for B. ascendens, indicating it to be a tetraploid species. Using *rbcL* sequence comparisons Hauk (1995) found *Botrychium ascendens* clustered with the lineage including *B. campestre* and *B. lineare* W. H. Wagner, suggesting this lineage contributed the chloroplast genome to *B. ascendens.* Using allozymes at a smaller number of loci (6) than the current study, Hauk and Haufler (1999) found the non-chloroplast genome of *B. ascendens* clustered with *B. crenulatum* W. H. Wagner, thus suggesting an allopolyploid origin of *B. ascendens* through ancient hybridization between *B. campestre*/*B. lineare* and *B. crenulatum.* In this paper we examine the variation of the Newfoundland population, and compare it with the morphology and allozymes of *B. ascendens* and related species of known provenance from elsewhere in North America, to confirm the identity of the Newfoundland plants and provide further evidence of their origin. We also present morphological characters useful in separating *B. ascendens* from *B. campestre* and *B. lineare.*

MATERIALS AND METHODS

Plant collection.—Plants of *Botrychium ascendens* were obtained from Sandy Cove on Fogo Island in Newfoundland on June 26, 2001. Plants representing the observed variation were collected by cutting leaves at ground level and storing them in plastic containers in an ice chest until processing. The plants were divided into groups, one for immediate pressing and one maintained fresh for enzyme electrophoresis. After samples for electrophoresis were removed from the common stalk, the latter were also pressed for herbarium vouchers. Vouchers of all plants in the study are deposited in the Ada Hayden Herbarium of Iowa State University (ISC).

Voucher specimens are labeled: CANADA: **Newfoundland**: Fogo Island, Sandy Cove, 7 July 1985, *Britton 10671 & Anderson* (MICH); Fogo Island, Sandy Cove, elev. ca. 3 m, 49° 42.5′ N, 54° 5.0′ W, 26 June 2001, *Zika 16334* (CAN, ISC, MICH, MT, NFM, WTU). The population is quite local and restricted to 80 meters of low sand dunes southwest of Route 334, at Sandy Cove, by a sandy beach near Tilton. About 150 stems were seen on 26 June 2001. The fern's associates included *Carex nigra* (L.) Reichard, *Festuca rubra* L., *Achillea, Linnaea, Fragaria, Taraxacum, Aralia nudicaulis* L., *Ranunculus acris* L., *Equisetum arvense* L., and *Botrychium lunaria.*

Allozyme electrophoresis.—From an ongoing study of all moonwort *Botrychium* by Farrar, we constructed genetic profiles obtained through starch-gel enzyme electrophoresis for species relevant to this study. The source of genetically analyzed plants is listed in Table 1. The number of individuals sampled per site ranged from 1 to 92 depending on population size. The average number of plants per site was 10.6.

Single leaves were cut at ground level and kept cool until processing. Approximately one-centimeter segments were removed from the common stalk

TABLE 1. Source of collections used in genetic analysis, including the number of sites sampled and total number of plants analyzed. Details of site locations can be obtained from Farrar.

Botrychium	State or Province	# Sites	# Plants
ascendens	Alaska	6	84
	California	1	12
	Montana	5	19
	Nevada	1	3
	Newfoundland	1	10
	Oregon	2	34
	Washington	3	24
campestre	Alberta	1	3
	Iowa	5	47
	Michigan	1	16
	Minnesota	7	74
	Montana	4	17
	South Dakota	3	38
	Wyoming	1	1
crenulatum	Alberta	1	6
	California	9	41
	Montana	5	19
crenulatum	Nevada	3	15
	Oregon	5	27
	Utah	2	25
lineare	Alaska	1	2
	Colorado	2	12
	Montana	5	34
	Oregon	2	15
	South Dakota	3	11
	Washington	1	1
	Wyoming	3	11
	Yukon	1	1
*lunaria**	Alaska (coastal)	18	476
	Colorado	2	51
	Michigan	3	25
	Ontario	1	15
	Washington	5	16
Total		113	1185

*The common American genotype differs genetically from European *B. lunaria* suggesting it could be described as a new species (Stensvold 2008).

(petiole) and ground with mortar and pestle in a phosphate-polyvinypyrrolidone extraction buffer (Cronn *et al.*, 1997). Grindates were stored at −70°C until used, at which time they were spun at 12,000 rpm for two minutes to produce a clear enzyme-containing supernatant. The extracts were absorbed onto 2 × 8 mm wicks cut from Whatman 3μ chromatography paper (Whatman International, Maidstone, UK). Allozyme variation was determined via horizontal starch-gel electrophoresis. Gel (11%) buffers and stain recipes followed Soltis *et al.* (1983).

Ten enzyme systems stained for 22 putative loci found to be informative within the genus. System 7 (Soltis *et al.*, 1983) was used to resolve triosphosphate isomerase (Tpi-1, Tpi-2), aspartic acid transaminase (Aat-1, Aat-2, Aat-3, Aat-4), and phosphoglucoisomerase (Pgi-2). System 9 was used to resolve malate dehydrogenase (Mdh-1, Mdh-2, Mdh-3, Mdh-4), 6-phosphogluconate dehydrogenase (6-Pgd-1), and phosphoglucomutase (Pgm-1, Pgm-2). System 11 was used to resolve aconitase (Aco-1, Aco-2), diaphorase (Dia-1, Dia-2, Dia-3, Dia-4), isocitrate dehydrogenase (Idh-1), and shikimic dehydrogenase (Skdh-1).

Analysis of spores.—Spore sizes were measured as the longest diameter of a minimum of 20 spores from each of ten plants and compared with published values for *Botrychium ascendens* and related diploid species.

Morphological comparisons.—Leaf morphology for Newfoundland plants was compared with *Botrychium ascendens* from western states and with *B. campestre* and *B. lineare* from all sites listed in Table 1. Illustrations were prepared to capture the range of morphology for each species.

RESULTS

Allozyme electrophoresis.—Twenty enzyme loci were variable among the compared taxa (Table 2). The allelic composition of *Botrychium ascendens* from Newfoundland was identical to the combined expression of western *B. ascendens* populations from Oregon, Washington, Montana, Nevada, California and Alaska (Table 2). Plants of identical genotype were present within the Newfoundland populations and the type locality of *B. ascendens* in Oregon. *Botrychium ascendens* from all sites displayed fixed heterozygosity at seven of the 22 loci examined. Four additional loci varied among populations for fixed heterozygosity, with some plants expressing only one of the alleles of the heterozygous condition. At all loci, the alleles present in *B. ascendens* are also present in *B. lineare*, *B. campestre* and/or *B. crenulatum*. At five loci, *Tpi-1*, *Tpi-2*, *Mdh-1*, *Mdh-2*, and *Skdh-1*, the single allele present in the American genotype of *B. lunaria* (L.) Swartz is not present in *B. ascendens*.

Analysis of spores.—Average spore size of *Botrychium ascendens* plants from Newfoundland was 41 μm and ranged from 39 to 44 μm. Wagner and Wagner (1986) reported spore sizes of 44–47 μm for *B. ascendens* from western plants. Wagner and Wagner (1986) reported a range of 34–38 μm for *B. campestre*. Wagner and Wagner (1994) did not include spore size in their description of *B. lineare*. Our measurements of spores of *B. lineare* averaged 36.2 μm and ranged from 35 to 39 μm.

Morphological comparisons.—Leaf morphology for Newfoundland plants was compared with *Botrychium ascendens* from western states and with *B. campestre* and *B. lineare*. Small Newfoundland plants closely resemble *B. lineare* and *B. campestre* (Figs. 1–3). Larger plants display features more typical of western plants of *B. ascendens* (Figs. 4–5). Typical plants of *B. ascendens* display a morphology intermediate between *B. campestre*/*lineare* and *B. crenulatum* (Fig. 6).

TABLE 2. Allozyme composition of *Botrychium ascendens* and putative parents. The number of individuals of each species analyzed is indicated (in parentheses). Alleles for each locus are represented by numbers. Numbers separated by a comma are alternate alleles expressed in different individuals. Numbers joined by "+" are two alleles present (fixed) in all individuals of the species or population. In *Aco*, *Idh*, and *Pgi*, *B. ascendens* displays fixed heterozygosity in most populations, but in some populations displays only one of the alleles present in the heterozygous state.

Allozyme locus	*B. campestre* (197)	*B. lineare* (91)	*B. ascendens* (186)	*B. crenulatum* (168)	*B. lunaria* (583)
Aat-1	2	2	1 + 2	1, 2	2
Aat-2	3	3	3	3	3
Aat-3	2	2, n	2	n	n
Aat-4	3	3	2 + 3	2, 3	2, 3
Aco-1	2	2	1, 1 + 2	1, 2	1
Aco-2	1	3	3, 1 + 3	1, 3	1, 3
Dia-1	4	2, 4	2 + 4	2	2
Dia-2	1	1	1	1	1
Dia 3	2, 3	2, 3	2 + 3	3	3
Dia-4	4	4	4	n	n
Idh	2, 3	1, 2	2, 2 + 3, 3, 2 + 4	3, 4	2
Mdh-1	3	1, 3	1 + 2	2	4
Mdh-2	3	3	2 + 3	2	5
Mdh-3	2*	1*, 2*	2*	2*	2
Mdh-4	2	2	2	n	n
6Pgd	1	1	1 + 5**	5	5
Pgi-2	2	2	2, 2 + 4, 4	4, 5	4
Pgm-1	1***	1***	1***	1	1
Pgm-2	2, 4	2	2	2	2
Skdh	1, 2	1	1	1	2
Tpi-1	3	3	3	3	4
Tpi-2	3	3	3	3	4

*In these species an additional spot of unknown origin always trails the principal allelic spot at a uniform distance regardless of the allele.

**Activity of the homodimers varied among populations; in some, only the heterodimer expressed normal activity.

***In these species an additional spot, possibly from a locus duplication, always precedes the principal allelic.

n = null, i.e., no allele expressed.

DISCUSSION

Moonwort ferns of *Botrychium* subgenus *Botrychium* are notoriously difficult to identify with certainty by morphological characters alone. They have been appropriately referred to as cryptic species (Hauk and Haufler, 1999). Because of their small size and simple morphology, differences between species are subtle and tend to be statistical rather than absolute. This problem is compounded in allotetraploids, in which the ranges of morphological characters overlap those of the parental diploids.

In contrast to morphology, genetic markers clearly define species of moonworts and, for allotetraploids, provide evidence of the ancestral diploid

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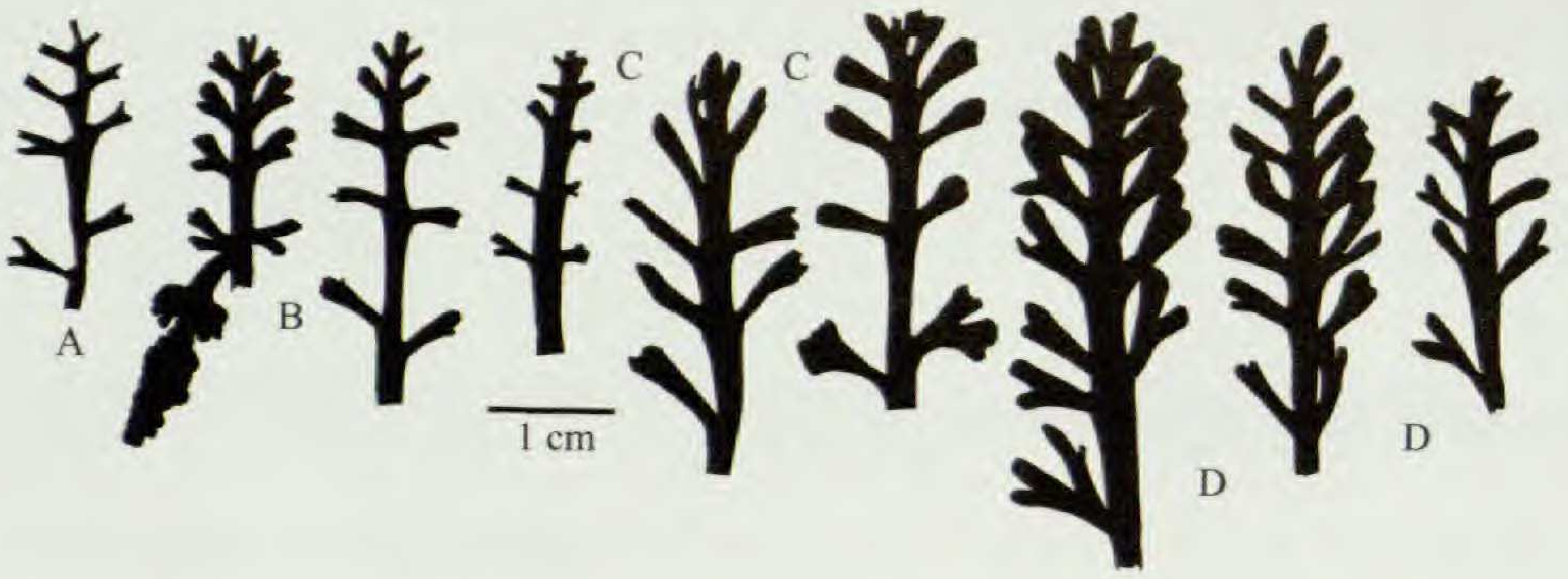

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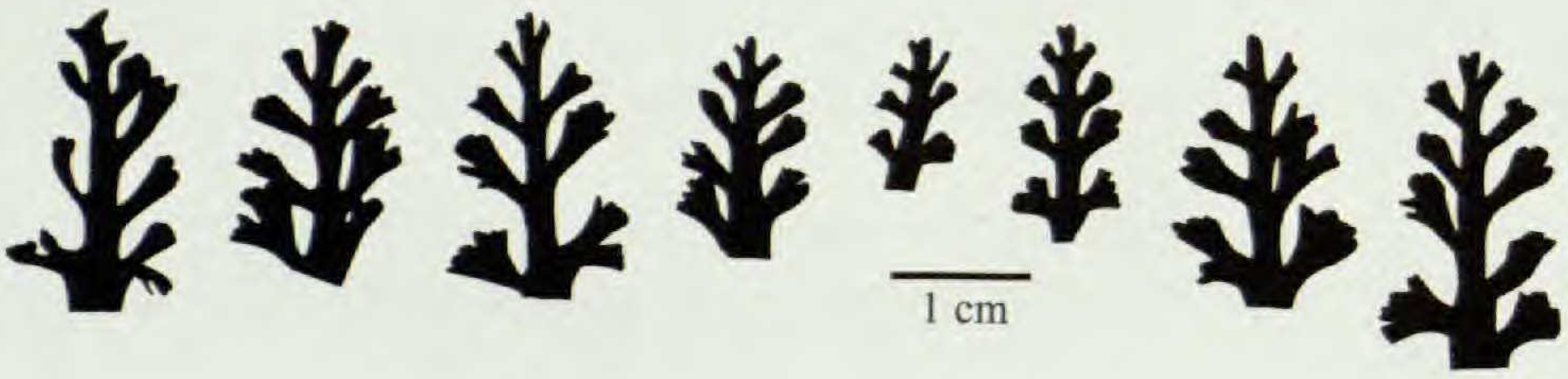

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FIG. 1. *Botrychium lineare* trophophores, showing typical slender pinnae with margins essentially straight or narrowing to base, notched or forked at tip. A. Washington (*Kirk 200 et al.* WTU). *B.* Type locality, Oregon (*Farrar 3918, E2235* ISC). C. Oregon (left to right: *Zika 12906* OSC; *Wagner 81128 & Wagner* MICH; *Zika 11353A* OSC). D. Colorado (left to right: *Farrar 3794, 3797, 3801* ISC). FIG. 2. *Botrychium ascendens* trophophores. Population variation showing narrow extremes from

species involved in their formation. In our comparison of genetic profiles determined for 22 loci in 10 enzyme systems, plants of *B. ascendens* from Newfoundland were consistent with those obtained for western plants from throughout the range of *B. ascendens*, including the type locality in northeastern Oregon. They differed from some (but not all) western populations only in not possessing fixed heterozygosity at *Aco-2*. This character state is also present in some populations in Oregon and Alaska. As with all plants of *B. ascendens*, in addition to possessing fixed heterozygosity, Newfoundland plants differed from both *B. lineare* and *B. campestre* in possessing many alleles not present in either of those species.

Morphological comparison of the Newfoundland *Botrychium ascendens* with *B. campestre* and *B. lineare* is useful in field and herbarium identifications. Slender plants from Newfoundland with narrow pinnae resemble *B. lineare* (Fig. 1), but tend to have broadened distal segments with more dentate outer margins (Fig. 2). The larger Newfoundland plants (Fig. 4) compare favorably with western specimens of *B. ascendens*, including plants from the type locality. They show somewhat more highly divided segments than the type collection of *B. ascendens* from Wallowa Co., Oregon (*W. H. Wagner 83363 et al.* MICH). However, this variation in morphology is comparable to larger specimens collected subsequently from the type locality, as well as material collected in Washington and Alaska (Fig. 5). There also exist many individuals (not illustrated) with morphology intermediate and transitional between the plants depicted in Fig. 2 and Fig. 4 from Newfoundland, showing increasingly broad and fan-shaped proximal pinnae. Thus, as the plants increase in size, their morphology trends away from the characteristic narrowness of *B. lineare*.

In comparison to *Botrychium campestre*, *B. ascendens* has less fleshy central axes and has basal pinnae that are more uniformly and broadly fan-shaped. Pinnae of *B. ascendens* are also more evenly spaced along the rachis of the trophophore and are more regularly and more deeply cleft into spreading lobes (Figs. 2, 4, 5). *Botrychium campestre* (Fig. 3) sometimes shows partial fusion of adjacent pinnae, and a broadly decurrent basal margin to some of the basal pinnae, features absent in *B. ascendens*. The outer margins of pinnae and pinnae lobes are dentate in *B. ascendens* and usually entire to crenulate in *B. campestre*. The basal pinnae are usually the largest in *B. ascendens* and

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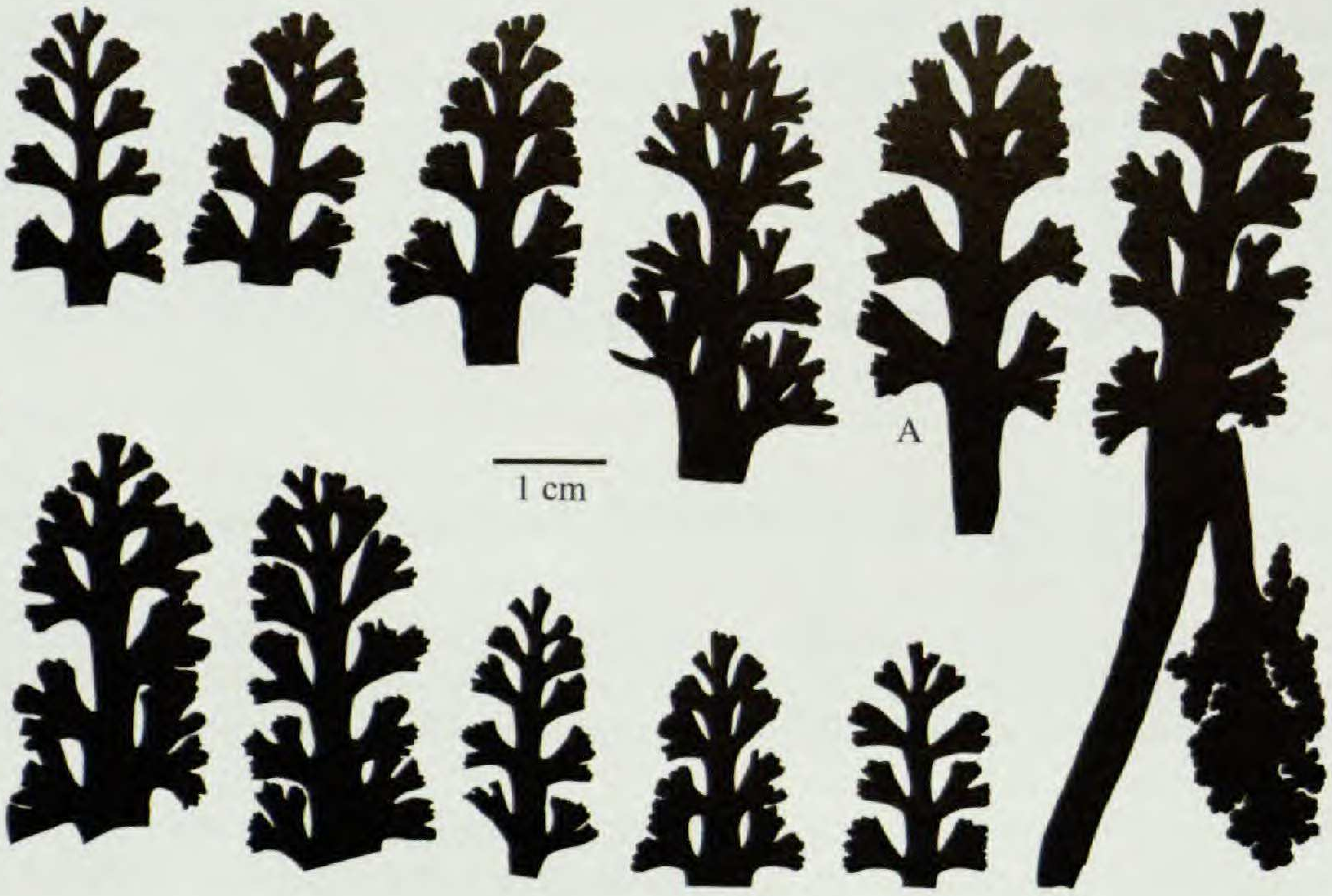
small plants in Newfoundland. Distal pinnae slender, as in *B. lineare*, but proximal pinnae broadened and with outer margins tending to be dentate (*Zika 16328* WTU).

FIG. 3. *Botrychium campestre* trophophores. Larger plants showing fleshy rachis, decurrent base of proximal pinnae, irregular and coalescing pinnae. Some smaller plants with more linear segments, approaching *B. lineare*, but pinnae apices more irregular. A. Michigan (*Wagner 85024 et al.* ISC). *B.* Michigan (*Wagner 85025 et al.* MICH). C. Michigan (*Wagner 85026 et al.* MICH). D. Michigan (*Wagner 85024 et al.* MICH). E. Minnesota (*Farrar 3495* ISC). F. Nebraska (*Farrar 86-6-2-3D* ISC). G. Minnesota (*Farrar 3497* ISC). H. Iowa (*Farrar 952* ISC). I. Type locality, Iowa (*Farrar 3484* ISC, on left; *Farrar 86-5-31-1* ISC).



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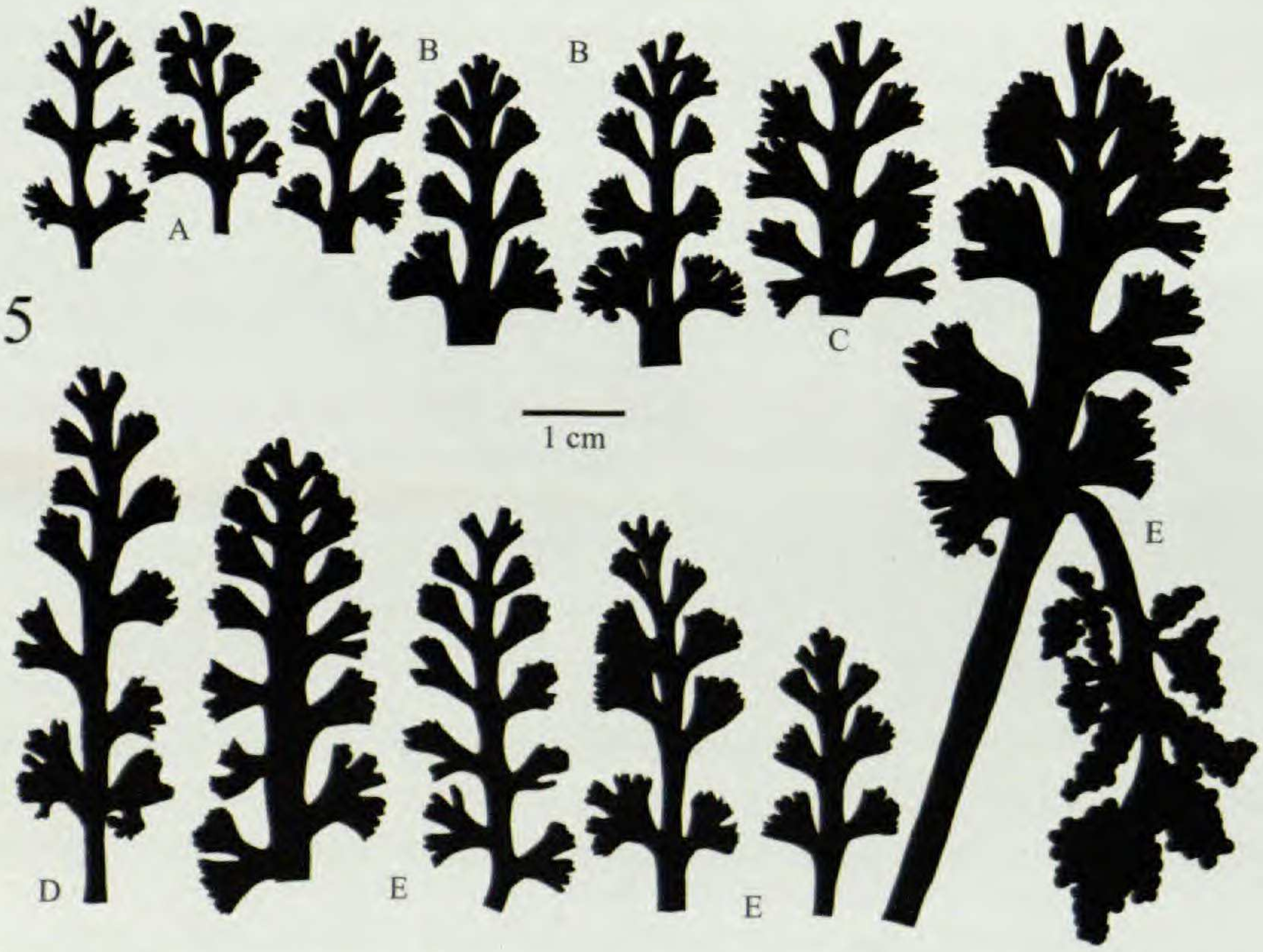

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FIG. 4. *Botrychium ascendens* trophophores. Population variation showing large and deeply divided pinnae from Newfoundland. A. *Britton 10671 & Anderson* (MICH); all others *Zika 16328* (WTU).
FIG. 5. *Botrychium ascendens* trophophores. Typical plants from western North America. A. Type collection, Oregon (*Wagner 83363 et al.* MICH). *B.* Type locality, Oregon (*Zika 17090* OSC). C. Washington (*Larson 254* WTU). D. Alaska (*Smith s.n.* ALA). E. Washington (*Buege s.n.* WTU).

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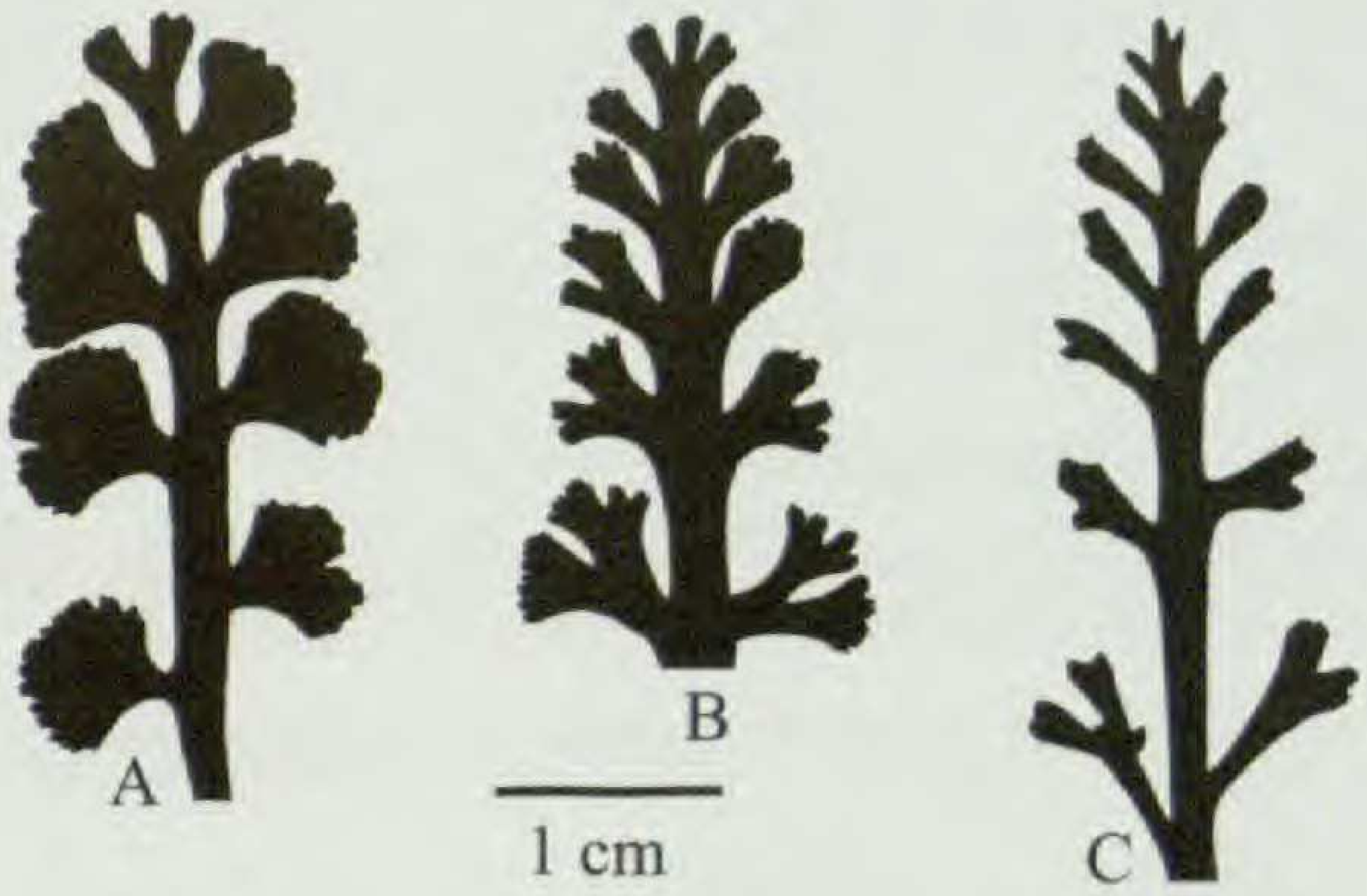

FIG. 6. Proposed allopolyploid parentage of *Botrychium ascendens*. A. *Botrychium crenulatum* (*Farrar 2031* ISC). B. *Botrychium ascendens* (*Farrar 1818* ISC). C. *Botrychium lineare* (*Farrar 3921* ISC).

frequently bear a few sporangia, whereas a non-basal pair is usually largest in *B. campestre*, and trophophore pinnae seldom bear supernumerary sporangia. Some *B. campestre* show basal pinnae erect in the axil between trophophore and sporophore, a feature not observed in *B. ascendens*. In large, well-developed plants, the non-basal pinnae of *B. ascendens* often tend to have a broader outer margin.

An additional feature helpful in separating *Botrychium ascendens* from *B. campestre* and *B. lineare* is the length of the sporophore stalk, which, in *B. ascendens*, reaches 1/3 to 2/3 the length of the trophophore. In *B. campestre* and *B. lineare* the sporophore stalk is usually ¼ or less the length of the trophophore. It must be noted, however, that this character is useful only in mature plants when (at spore release) the sporophore stalk has ceased elongation. The diploid species, *B. campestre* and *B. lineare*, also have smaller spores (34–39 μm) than does tetraploid *B. ascendens*, from Fogo Island and elsewhere (39–47 μm). All three can bear inconspicuous asexual reproductive gemmae on their subterranean stems (Johnson-Groh *et al.*, 2002). Both *B. campestre* and *B. lineare* have been reported from northeastern North America (Kartesz, 1999; Hinds, 2000).

Recombinational heterozygosity at a given locus segregates in meiosis and recombines in syngamy to produce a predictable proportion of individuals in a population that are homozygous. Occurrence of heterozygosity in all individuals of a population at a large proportion of loci is best explained as non-recombinational or fixed heterozygosity resulting from allopolyploidy. Plants of *B. ascendens* from Newfoundland and elsewhere display identical fixed heterozygosity at seven of 22 loci examined, and, in some populations, at four additional loci. Consistent with its chromosome number of n = 90 (Wagner and Wagner, 1986), this strongly indicates that *B. ascendens* is an allotetraploid derived through ancient hybridization between two diploid species. This is also supported by morphological evidence.

The allelic composition of *Botrychium ascendens* matched an expected composition resulting from hybridization between either *B. campestre* or *B. lineare* and *B. crenulatum* (Table 2). Of the 11 loci displaying only a single allele in *B. ascendens*, this allele is present in both of these putative parental lines (8) or null in *B. crenulatum*. In the loci displaying fixed heterozygosity, *B. crenulatum* possesses at all loci the alleles necessary to create, in combination with *B. campestre/lineare*, the allelic combinations present in *B. ascendens*. This includes an allele at *Mdh-2* that, among diploid species of *Botrychium*, is found only in *B. crenulatum*. Close genetic similarity between *B. lineare* and *B. campestre* does not allow differentiation between these two species as to the most likely parent, but the *B. campestre/lineare* lineage is strongly implicated.

Botrychium lunaria is the only diploid species other than *B. crenulatum* possessing the broad pinna shape predicted for the non-*B. campestre/lineare* parent of *B. ascendens*. The American genotype of *B. lunaria* is homozygous at five loci (*Tpi-1*, *Tpi-2*, *Mdh-1*, *Mdh-2*, *Skdh-1*) for alleles not present in *B. ascendens*, and fails to provide the complimentary allele missing from *B. campestre/lineare* at an additional locus (*Aat-1*). The European genotype of *B. lunaria* is similar to that of *B. crenulatum*, but does not contain the *Mdh-2* allele uniquely present in *B. crenulatum* and *B. acendens* (Stensvold 2008).

The morphology of *Botrychium ascendens* is also consistent with parentage of *B. crenulatum* and *B. lineare* (Fig. 6). The ascending pinnae and their tendency to be bifurcate likely reflect tendencies inherited from *B. lineare*. The dentate outer margins of *B. ascendens* pinnae reflect the intermediacy between the crenulate margins of *B. crenulatum* and the entire margins of *B. lineare*.

Botrychium lineare and *B. campestre* occur in both eastern and western North America. *Botrychium crenulatum* is known only in western North America, ranging in western mountains from southern California and Nevada to southern British Columbia and eastward to central Alberta. If these patterns represent distributions of the parent species at the time of the formation of *B. ascendens*, eastern Canadian populations of *B. ascendens* likely result from migration of the species from western to eastern North America. *Botrychium ascendens* is a fertile tetraploid. Gametophytes from a single spore are capable of producing sporophytes through self-fertilization in *Botrychium*, thus long distance migration via single spores is possible. The habitat of the plants in Newfoundland is remarkably similar to the back-beach sand dune habitat of *B. ascendens* and other *Botrychium* species in south coastal Alaska. A single collection of *B. ascendens* from the south shore of Hudson Bay (*Moir 1444* CAN) suggests the possibility of a broader occurrence of *B. ascendens* in similar habitats across northern Canada.

Both morphological and genetic evidence confirm that *Botrychium ascendens* is extant in the province of Newfoundland, in Fogo District, Fogo Island, off the northeast shore of the island of Newfoundland.

We hope this discovery in Newfoundland will encourage botanists in eastern Canada to search for additional extant populations of *Botrychium ascendens*, a small and inconspicuous species that possibly has been overlooked in coastal and other habitats.

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